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CD80 and CD86, but not CD154, augment DNA vaccine-induced protection in experimental bovine tuberculosis

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Abstract

DNA vaccination is known to elicit robust cellular and humoral responses to encoded antigen. The co-administration of costimulatory molecules CD80 (B7-1), CD86 (B7-2) and CD154 (CD40L) has been shown to enhance immune responses in several murine models. The role of specific costimulatory molecules in non-rodent species remains incompletely characterized. In these studies, we demonstrate that the co-administration of CD80 and CD86, but not CD154, to an existing candidate subunit DNA vaccine (ESAT-6) against bovine tuberculosis, enhances protection after aerosol challenge with virulent *Mycobacterium bovis*. Additionally, we have shown that vaccination with *M. bovis* BCG is protective against tuberculosis following aerosol challenge in cattle. Two independent trials were conducted in cattle to determine the adjuvant effect of encoded antigen + CD80/CD86 and directly compare the adjuvant activities of CD80/CD86 to those of CD154. Co-administration of either CD80/CD86 or CD154 enhanced ESAT-6-specific IFN-γ responses as compared to animals vaccinated with ESAT-6 DNA alone. However, following aerosol challenge, only animals vaccinated with CD80/CD86 possessed decreased pathology of the lungs and associated lymph nodes, as measured by gross examination, radiographic lesion morphometry and bacterial recovery. Collectively, these results demonstrate that the co-administration of costimulatory molecules with a protective antigen target enhances bovine immune responses to DNA vaccination, and that CD80/CD86 is superior to CD154 in augmenting DNA vaccine-induced protection in experimental bovine tuberculosis.

Keywords: Costimulatory molecules; CpG ODN; Adjuvant

1. Introduction

DNA vaccination has been shown to generate cell-mediated and humoral immune responses to encoded antigen in several different species [1–4]. In addition to the induction of immunity, DNA vaccination offers several advantages when compared to conventional vaccines, such as ease of production, stability, cost effectiveness and the overall flexibility of the vaccine platform [1,2,4,5]. DNA vaccines are also attractive candidates for disease prevention due to their ability to potentially enhance immunity via $CD8^+$ T cells through the MHC class I pathway [1,2,4]. Several studies in mice have shown that DNA vaccination provides some level of protective immunity against infectious agents [2,6].

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While these experiments have demonstrated the utility of DNA vaccination as a means of eliciting cell-mediated and humoral responses, it has been difficult to generate comparable immune responses to DNA vaccines in humans and large animals [3,5,7]. Progress towards successful DNA vaccines in non-rodent species has been hampered by several factors. DNA vaccination of large animals often requires relatively large amounts of plasmid DNA administered in multiple doses [8–10]. Previous studies have also shown that individuals vaccinated with DNA are relatively low responders [8–10]. Additionally, there remains relatively little efficacy data on DNA vaccines following infectious challenge due to the relatively high cost of large animal studies. Recently, studies in cattle have shown that co-administration of plasmid-encoded FLT-3L and GM-CSF enhanced bovine $CD4^+$ T cell responses to DNA vaccination [11]. Similarly, plasmid-encoded bovine CD40L (CD154) used as an adjuvant and vaccine-targeting molecule, increased antibody responses to bovine herpesvirus 1 glycoprotein D in sheep [12]. CpG oligodinucleotides (ODN), when used in the correct species-specific context [13], have been shown to possess adjuvant activity by inducing the production of inflammatory cytokines from bovine peripheral blood mononuclear cells (PBMC) in vitro [14]. Thus, the co-administration of molecular adjuvants may represent a means of enhancing immune responses to DNA vaccination in non-rodent species.

While previous studies in outbred large animal species have concluded that it is possible to enhance immune responses to DNA vaccination using various strategies, the role of costimulatory molecules in enhancing immunity in ruminants remains incompletely characterized. The objective of this study was to evaluate the ability of plasmid-encoded bovine CD80 and CD86 to augment DNA vaccine-induced cell-mediated immunity in a model of experimental bovine tuberculosis. It has been demonstrated previously that CD28 signaling is required for immune responses to DNA vaccination [15]. In rodents, DNA vaccination using a co-linear construct encoding CD86 and a suboptimal influenza nucleoprotein epitope resulted in an enhancement of cytotoxic T lymphocyte (CTL) activity that was >80% of the response seen with influenza virus-infected controls [16]. Similarly, mice vaccinated with plasmid encoding HIV-1 envelope protein possessed enhanced IFN-γ and CTL responses with the co-administration of a separate plasmid encoding CD86, thus revealing that adjuvant effects with costimulatory molecules can be seen whether or not the sequences are co-expressed by the same construct [17,18]. Enhancement of immune responses via CD86 costimulation has also been shown in chimpanzees, which developed more robust T cell responses to HIV-1 envelope protein after receiving CD86-encoding plasmid [17]. Additional studies in mice have shown that the co-administration of encoded CD80 may also be used to enhance CTL responses to DNA vaccination [19,20]. To date, few if any studies have been conducted in cattle regarding the use of DNA-encoded CD80 and CD86 to enhance vaccine responses.

After assessing the potential of CD80 and CD86 as molecular adjuvants, we directly compared the effects of co-administering plasmid-encoded CD154 against encoded CD80 and CD86. CD154 is expressed rapidly on T cells following activation [21,22] and has a role in the generation of both cell-mediated and thymus dependent humoral responses, as well as regulation of antigen presenting cell (APC) activity [23,24]. Ligation of CD40 by CD154 stimulates immature APCs to differentiate, produce cytokines and upregulate the expression of costimulatory molecules [23,24], thus creating a more efficient APC. Several rodent studies have examined the effect of plasmid-expressed CD154 on DNA vaccination. Mendoza et al. [25] found that the co-administration of CD154 enhanced IgG2a production and CTL responses to plasmid-encoded β-galactosidase, suggesting a role in augmenting cell-mediated responses. Similarly, mice vaccinated with a HIV DNA vaccine and a CD154 expression plasmid possessed antigen-specific increases in IgG2a and IFN-γ production and CTL activity [26]. The latter study also determined that co-administration of CD154 increased antigen-specific Th2 responses. The aforementioned study in ruminants regarding CD154 and DNA vaccination revealed the potential of CD154 as an adjuvant to enhance humoral responses [12], but to date, little data has been published examining the effect of CD154 on cell-mediated responses in large animals following infectious challenge.

Challenge with *Mycobacterium tuberculosis* or *M. bovis* represent established models of infection that require cell-mediated immune responses for protective immunity. Roles for $CD4^+$ and $CD8^+$ T cells have been delineated in mice, with Th1-mediated responses correlating with protection [27]. The early secretory antigenic target-6kDa (ESAT-6) protein is recognized by bovine T cells early during *M. bovis* infection [28], resulting in the release of IFN-γ [29,30], making it an attractive candidate for vaccine development. ESAT-6 DNA vaccination of mice resulted in significantly reduced bacterial recovery following challenge with *M. tuberculosis* [31]. To test the utility of these combined approaches in large animals, we vaccinated cattle with an ESAT-6 DNA vaccine, CpG ODN and plasmid-encoded costimulatory molecules using a prime and single boost strategy. Collectively, our results suggest that a DNA vaccine composed of plasmids encoding CD80 and CD86 administered with CpG ODN provides a practical means of generating improved immune responses in large animals.

2. Materials and methods

2.1. Plasmid DNA, CpG ODN and rESAT-6

Plasmid encoding bovine CD80 was kindly provided by Dr. Chris J. Howard (Institute of Animal Health, Compton, UK). Plasmid encoding bovine CD86 was kindly provided by Dr. Keith R. Parsons (Institute of Animal Health, Compton, UK). The construction of bovine CD80 and CD154 plasmids has been described previously [32,33]. The plasmid for

bovine CD86 encodes the majority of the open reading frame described in GenBank (accession AJ291475 and AY533858) to 1092 nucleotides. The clone used for these studies lacks seven amino acids in the N-terminal region of the protein (residues 22–28). Expression of bovine CD80, CD86 and CD154 was confirmed in transfected COS-7 cells by flow cytometry (data not shown) as previously described for dendritic cells (DC) [34]. Transfections were performed according to the manufacturer's instructions (Lipofectamine, Invitrogen, Carlsbad, CA). Plasmid encoding ESAT-6 was constructed by amplifying the complete open reading frame of ESAT-6 from pA using forward primer (5′-GAGAGATCTCATGACAGAGCAGCAGTGGAATTTC-3′) and reverse primer (5′-GGCAGATCTCTATGCGAACATCCCAGTG-3′) as described previously [35]. The amplified fragment was digested with *Bgl*II and was then inserted into the *Bam*HI site of plasmid VR1012 (Vical Inc., San Diego, CA) to generate pISM411. Plasmid DNA for immunization was prepared by transforming plasmids into competent *Escherichia coli* using the pcDNA3.1 Directional TOPO Expression cloning kit (Invitrogen, Carlsbad, CA). Gene inserts were confirmed by automated DNA sequencing (DNA Core Facility, University of Missouri). Plasmid DNA was purified using the Qiagen Plasmid Giga kit (Qiagen, Valencia, CA) according to the manufacturer's instructions.

CpG ODN were manufactured by Oligos Etc. (Wilsonville, OR). ODN 2006 (TCGTCGTTTTGTCGTTTGTCGTT) has been shown to stimulate bovine PBMC in vitro [14] and augment cellular and humoral immune responses in vivo [36]. ODN 2006 contains a phosphothioate-modified backbone that renders it nuclease-resistant. CpG ODN were stored as lyophilized aliquots at −80 °C until use and then were resuspended in sterile PBS.

Purified rESAT-6 was obtained as described previously [35]. Briefly, the coding sequence for ESAT-6 was obtained by PCR amplification and cloned into pTrcHis B (Invitrogen Corp., San Diego, CA) to form pISM403. Induction of *E. coli* TOP 10 cells containing pISM403 was accomplished by the addition of 1 mM isopropyl-thio-β-D-galactopyransoide and incubating for 6–8 h. The recombinant protein was purified by metal chelate chromatography as described [35]. Purity was assessed using sodium dodecyl sulfate polyacrylamide gel electrophoresis and immunoblotting with anti-Xpress antibodies (Invitrogen).

2.2. Bacteria

M. bovis strain 1315 and attenuated *M. bovis* BCG strain Pasteur were grown in Middlebrook 7H9 media supplemented with 10% oleic acid–albumin–dextrose complex (Difco, Detroit, MI) plus 0.05% Tween 80 (Sigma, St. Louis, MO) as described previously [37]. *M. bovis* 1315 was originally isolated in 1995 from an infected white-tailed deer [38]. Challenge and vaccination inocula consisted of mid-log growth phase mycobacteria. Enumeration of bacilli was confirmed using serial dilution plate counting on Middlebrook 7H11 selective media (Becton Dickinson, Cockeysville, MD).

2.3. Animals and immunizations

2.3.1. DNA vaccine trial #1

Eighteen castrated male mixed breed cattle, 400–600 lbs, were selected based on their negative reactivity to purified protein derivative (PPD) from *M. avium* (PPDa) and *M. bovis* (PPDb). Briefly, whole blood was incubated for 24 h in vitro in the presence of 20 μg/ml PPDb and PPDa (Biocor Animal Health, Omaha, NE). Supernatants were then harvested and PPDb-specific IFN-γ production was determined using the BoviGam ELISA kit (Biocor Animal Health). Animals negative for prior exposure to mycobacteria were then randomly assigned to the following four experimental treatment groups: (1) *M. bovis* BCG strain Pasteur vaccination (n = 4); (2) ESAT-6 DNA vaccination (n = 5); (3) ESAT-6 + CD80/CD86 DNA vaccination (n = 4); (4) pcDNA3.1 (empty plasmid) + CD80/CD86 control DNA vaccination (n = 5). DNA vaccinates were immunized intramuscularly (IM) with 2 mg of total plasmid DNA and with 1 mg of ODN 2006 emulsified in 2 ml of IFA in the left mid-cervical region. Cattle received an identical booster dose of vaccine 20 days post-prime. BCG animals were vaccinated with a single dose of 4.0×10^7 colony forming units (CFU) *M. bovis* BCG strain Pasteur administered IM in the left mid-cervical region at the time DNA vaccinates received their booster doses. All immunizations were conducted at the University of Missouri, Columbia. Immunizations were conducted in accordance with the guidelines of the institutional animal care and use policies of the University of Missouri, Columbia.

2.3.2. DNA vaccine trial #2

Twenty cattle (females and castrated males), 400–600 lbs, mixed breed cattle from the same herd, were selected based on their negative reactivity to purified protein derivative from *M. avium* (PPDa) and *M. bovis* (PPDb), as described in DNA vaccine trial 1. Naïve animals were then randomly assigned to the following five experimental treatment groups: (1) *M. bovis* BCG strain Pasteur vaccination (n = 4); (2) ESAT-6 DNA vaccination (n = 4); (3) ESAT-6 + CD80/CD86 DNA vaccination (n = 4); (4) ESAT-6 + CD154 DNA vaccination (n = 4); (5) pcDNA3.1 (empty plasmid) + CD80/CD86/CD154 control DNA vaccination (n = 4). DNA vaccinates were immunized intramuscularly with 2 mg of total plasmid DNA and with 1 mg of ODN 2006 emulsified in 2 ml of IFA in the left mid-cervical region. At the time of the first immunization, BCG animals were vaccinated once with a dose of 1.0×10^7 CFU *M. bovis* BCG strain Pasteur. All cattle, except for BCG vaccinates, received an identical booster dose of vaccine 20 days post-prime. All immunizations were conducted at the National Animal Disease Center, Ames, IA. Prior to experimentation, a protocol detailing procedures and animal care was approved by the Institutional Animal Care and Use Committee (IACUC).

2.4. Aerosol challenge of vaccinated cattle

Aerosol challenge of cattle with *M. bovis* was conducted at the National Animal Disease Center, Ames, IA. Cattle were sedated with 2.5 mg xylazine (Bayer Corp., Shawnee Mission, KS) given intravenously (IV). 3.6×10^3 CFU and 1.0×10^3 CFU of virulent *M. bovis* 1315 was administered to animals, in vaccine trials 1 and 2, respectively, using a modified commercially available Equine Aeromask (Trudell Medical International, London, Ont., Canada) aerosol delivery system comprised of a jet nebulizer (Whisper Jet, Marquest Medical Products, Englewood, CO), holding chamber and mask, that has been described previously [38]. This system generates a particle size of <5 μm, which is necessary to penetrate terminal airways of the lung. Following challenge, sedation was reversed with IV administration of 5 ml tolazoline (100 mg/ml) (Lloyd Laboratories, Shenandoah, IA). 95–100 days after challenge, infected cattle were euthanized. Euthanasia was conducted by IV administration of sodium pentobarbital (Sleepaway, Fort Dodge Laboratories, Fort Dodge, IA). Aerosol challenge and housing of cattle in biosafety level 3 (BL-3) facilities was conducted in accordance with the guidelines provided by the Association for the Assessment and Accreditation of Laboratory Animal Care International (AAALAC). Prior to experimentation, a protocol detailing procedures and animal care was approved by the Institutional Animal Care and Use Committee (IACUC).

2.5. IFN-γ ELISA

Whole blood cultures were performed in round bottom 96-well plates (200 μl/well) by mixing 190 μl heparinized blood with 10 μl of antigen in complete RPMI (cRPMI, RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids and 50 μg/ml gentamicin). Final concentration of antigen in wells for rESAT-6, PPDb and pokeweed mitogen (PWM) stimulation was 4, 5 and 1 μg/ml, respectively. Whole blood was cultured for 24 h in vitro at 37 °C. After incubation, supernatants were removed and stored at −80 °C prior to use. Supernatants were then assayed for IFN-γ production using the BoviGam ELISA kit in duplicate according to the manufacturers' instructions. Data are expressed as an ELISA index (experimental value/negative control (media only) × 100) or pg/ml. Concentrations (pg/ml) were based on linear regression from a standard curve. Recombinant bovine IFN-γ was kindly provided by Dr. Lorne Babiuk (University of Saskatchewan, Saskatoon, Canada).

2.6. IFN-γ Elispot assay

Three weeks following DNA vaccine boost, PBMC were isolated from heparinized blood by centrifugation over Accupaque (Accurate Chemical & Scientific Corp., Westbury, NY). ESAT-6-specific IFN-γ production was assayed using an ELISPOT assay as described [11,36] with some modifications. Monoclonal antibodies (Mab) CC302 and CC330 were kindly provided by Dr. Chris J. Howard. Briefly, triplicate wells of 96-well MultiScreen Immobilon-P plates (Millipore, Bedford, MA) were coated with 100 μl of 8 μg/ml anti-bovine IFN-γ MAb CC330 for 2 h at room temperature, and washed six times with PBS/0.05% Tween 20 (PBST). Plates were blocked with PBS containing 1% bovine serum albumin (BSA) for 2 h at room temperature. After washing with PBST, 2×10^5 PBMC were added in 100 μl volumes containing either cRPMI alone, rESAT-6 (4 μg/ml), or pokeweed mitogen (1 μg/ml). Plates were incubated for 36 h at 37 °C. Following incubation, plates were washed six times with PBST, once with distilled water, six times with PBST, then twice with PBS. Plates were then incubated for 2 h at room temperature with 100 μl of 5 μg/ml biotinylated mouse anti-bovine IFN-γ MAb CC302 diluted in PBS/1% BSA. Plates were washed and individual IFN-γ secreting cells stained using the HistoMark Biotin-Streptavidin Kit (Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD) according to the manufacturer's instructions. Plates were washed twice with distilled water and then dried. IFN-γ spot-forming cells were enumerated using a standard dissection microscope. For each animal, the mean number of spots of negative control wells was subtracted from the number of spots in test wells to determine the mean number of ESAT-6-specific IFN-γ spot-forming cells.

2.7. Lymphocyte proliferation assay

Heparinized whole blood was diluted 1:4 in cRPMI and 100 μl was added to 100 μl of antigen diluted in cRPMI in triplicate, at concentrations described above, in round bottom 96-well plates. Whole blood cultures were incubated at 37 °C for 7 days with the addition of 0.5 μCi [^{3}H] thymidine (specific activity, 6.7 Ci/mM, Amersham Life Science, Arlington Heights, IL) during the last 18–20 h of culture. Well contents were harvested onto glass fiber filters with a 96-well plate harvester (EG & G Wallac, Gaithersburg, MD), and the incorporated radioactivity measured by liquid scintillation counting. Treatments were run in triplicate and presented as mean cpm.

2.8. Histopathology

Lungs and mediastinal lymph nodes were subjected to a semi-quantitative gross pathology scoring system adapted from Vordemeier et al. [39] on coded samples. Lung lobes (left cranial, left caudal, right cranial, right caudal, middle and accessory) were subjected to the following scoring system: (0) no visible lesions; (1) no external gross lesions, but lesions seen upon slicing; (2) <5 gross lesions of <10 mm in diameter; (3) >5 gross lesions of <10 mm in diameter; (4) >1 distinct gross lesion of >10 mm in diameter; (5) gross coalescing lesions. Lymph node pathology was based on the following scoring system: (0) no necrosis or visible lesions; (1) small focus (1–2 mm in diameter); (2) several small foci; (3) extensive necrosis.

2.9. Radiographic lesion morphometry

To reinforce pathology findings, all lung lobes were radiographed after sacrifice using a MinXray machine (Model HF-100, Diagnostic Imaging, Rapid City, SD) with 3M Asymetrix Detail Screens and Ultimate 2000 film (3M Animal Care Products, St. Paul, MN). After development, radiographs were scanned to create digital images. Radiographic lesions were then identified, outlined and measured using Image Pro Plus (Media Cybernetics, Silver Spring, MD) software. Affected area was divided by total lung area then multiplied by 100 to determine percent affected lung. Results for individual animals are presented as the mean percent affected lung for all lung lobes.

2.10. Bacterial recovery

For the determination of bacterial load in the lung and lymph nodes, the right caudal and middle lung lobes and mediastinal lymph nodes were isolated from infected animals and weighed. Previous experiments have determined which lung lobes are most affected following aerosol challenge with 10^3 CFU of *M. bovis* 1315 for this duration of infection [38]. Lung lobes were homogenized in phenol red nutrient broth in a 1:1 (w/v) ratio in an industrial food processor (Hobart, Model No. HCM62-1, Gill Marketing Co., Crofton, MD) for 10 min. Mediastinal lymph nodes were similarly homogenized in phenol red nutrient broth using a commercially available blender. Logarithmic dilutions (10^0 to 10^{-9}) of homogenates were prepared in PBS and 100 μl aliquots plated over Middlebrook 7H11 selective agar plates (Becton Dickinson). Agar plates were incubated for 4 weeks at 37 °C for bacterial recovery. Data are presented as CFU per ml of mediastinal lymph node and lung homogenate.

2.11. Statistical analysis

Data was analyzed by Kruskal–Wallis one-way analysis of variance (ANOVA) using commercially available software (SigmaStat 3.0, SPSS, Chicago, IL). Differences between groups were considered significant at $P < 0.05$. Pairwise comparisons between groups were made using Student's *t*-test or a Mann–Whitney rank sum test. Differences between groups were considered significant at $P < 0.05$.

3. Results

3.1. Immunization with plasmid DNA encoding ESAT-6 results in enhanced IFN-γ production following in vitro stimulation

To determine whether ESAT-6 DNA vaccination generates effective cell-mediated immune responses, we compared the ability of bovine PBMC from different experimental treatment groups in DNA vaccine trial one to produce IFN-γ in response to antigenic stimulation following infectious challenge (Fig. 1a). It has been reported that IFN-γ is necessary for protection against tuberculosis [40]. Although not statistically significant, ESAT-6-specific IFN-γ production was enhanced approximately two-fold in cattle receiving ESAT-6 DNA vaccines as compared to control (vector only + CD80/CD86) immunized animals at 8 and 10 weeks post-challenge. Similarly, cattle vaccinated with ESAT-6 DNA produced higher levels of IFN-γ, when stimulated with PPDb in vitro (data not shown). Collectively, our results suggest that ESAT-6 DNA vaccination generates an immune response to ESAT-6 in cattle, which correlates with increased IFN-γ production in response to mycobacterial antigens following infection with *M. bovis*.

3.2. Costimulatory molecules enhance proliferative responses to rESAT-6 following infectious challenge

After experimental infection of cattle, we cultured whole blood with rESAT-6 to determine differences in proliferative

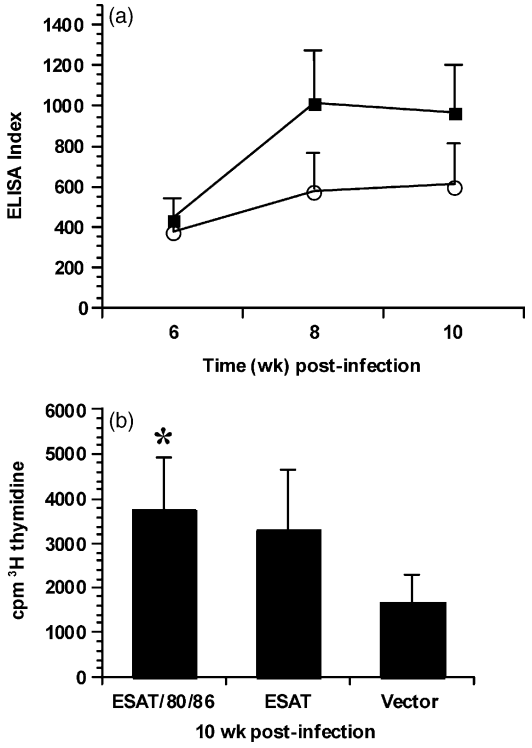

Fig. 1. Cell-mediated responses from ESAT-6 DNA vaccinates are enhanced following antigenic stimulation. Whole blood derived from animals receiving ESAT-6 DNA ($n = 9$) (■) or from control immunized animals ($n = 5$) (◯) was stimulated for 24 h in vitro with rESAT-6 (4 μg/ml) at 6, 8 and 10 week post-challenge. Supernatants were assayed for IFN-γ production using the BoviGam ELISA kit (a). Data represent the mean of duplicate cultures ± S.E.M. ESAT-6 + CD80/CD86 DNA vaccinated cattle ($n = 4$) exhibit enhanced proliferative responses to stimulation with rESAT-6 (4 μg/ml) as compared to responses of cattle vaccinated with control DNA (pcDNA3.1 + CD80/CD86) ($n = 5$) in whole blood cultures (b). The asterisk indicates a significant difference ($P < 0.05$) from responses of animals vaccinated with control DNA. Responses to media alone and pokeweed mitogen did not differ ($P < 0.05$) between groups. Data represent responses from 10 weeks post-*M. bovis* challenge (mean ± S.E.M.). Similar responses were detected at 8 weeks post-challenge (data not shown).

responses between treatment groups. Previous experiments have shown that T cells from infected humans, cattle and mice, proliferate in response to restimulation with rESAT-6 in vitro. The results presented in Fig. 1b reveal that ESAT-6 + CD80/CD86 DNA vaccinates show statistically significant proliferation of PBMC ($P < 0.05$) at 10 weeks post-infection in response to restimulation with rESAT-6 when compared to animals vaccinated with control DNA. Animals that received ESAT-6 DNA alone did not possess significant differences over control animals. This effect is most likely attributable to small group sizes and variation in individual animal responses. Similar results were seen with PPDb restimulation amongst treatment groups (data not shown). These results suggest that ESAT-6 + CD80/CD86 DNA vaccination results in an enhanced proliferative response to ESAT-6 as compared to control vaccinates.

3.3. ESAT-6 + CD80/CD86 DNA vaccinates show reduced pathology in the lungs following aerosol challenge with *M. bovis*

Previous experiments have determined the location of *M. bovis* after aerosol challenge (10^3 CFU) to be restricted to the lung and regional lymph nodes draining the lung [38]. To assess disease severity, lungs and lymph nodes were subjected to a previously described pathology scoring system for cattle infected with virulent *M. bovis* [39]. As shown in Table 1, BCG and ESAT-6 + CD80/CD86 DNA vaccinates had overall lower levels of lung pathology compared to other DNA vaccinates. According to the scoring of disease severity, control animals possessed the most severe pathology of the lung, followed by animals vaccinated with ESAT-6 DNA alone in vaccine trial one.

To reinforce the findings of this semi-quantitative scoring system, we next employed radiographic lesion morphometry to quantify lung lesions. Table 1 illustrates the findings of radiographic lesion morphometry from vaccination trial one and two. *M. bovis* BCG and ESAT-6 + CD80/CD86 DNA vaccinates show comparable levels of affected lung area after virulent *M. bovis* challenge for approximately 95 days in trial one. Cattle vaccinated with control DNA or ESAT-6 DNA alone, possessed the highest mean percent affected lung. Table 2 summarizes the percent affected lung on an individual lobe basis. ESAT-6 + CD80/CD86 DNA and *M. bovis* BCG vaccinates possessed the lowest mean percent affected lung on all of the lung lobes. Similar results were obtained regarding the number of radiographic lesions in the lung (Table 1). Low bacterial recovery was obtained from the lung homogenates of all treatment groups at this dose and time point (data not shown). Due to variation in individual responses, significant differences were not detected, although these results suggest that ESAT-6 + CD80/CD86 DNA vaccination achieves reduced pathology compared to ESAT-6 DNA vaccination alone following infectious challenge with *M. bovis*.

3.4. ESAT-6 + CD80/CD86 DNA vaccinates possess reduced pathology and bacterial recovery from mediastinal lymph nodes

Following challenge, mediastinal lymph nodes were subjected to a previously described pathology scoring system [39]. Results are summarized in Table 3. All ESAT-6 DNA vaccinates possessed significantly reduced pathology ($P < 0.05$) of the mediastinal lymph node compared to control animals in trial one. Additionally, ESAT-6 + CD80/CD86 DNA vaccinates possessed statistically lower lymph node weights ($P < 0.05$) compared to the other DNA vaccinates. To determine the protective efficacy of the DNA vaccines used in trial one, mediastinal lymph nodes of infected cattle

Table 1
Summary of pathological findings in the lung of *M. bovis*-infected animals

Vaccination group	Mean disease score[a]	Number of radiographic lesions	Animals with radiographic lesions	Affected lung (%)
DNA vaccine trial #1				
BCG	0.30 ± 0.30	1.50 ± 1.50	1/4	0.107 ± 0.107
ESAT/CD80/86	0.45 ± 0.26	1.25 ± 0.94	2/4	0.115 ± 0.087
ESAT	1.24 ± 0.61	8.00 ± 6.09	4/5	1.274 ± 1.013
Vector	1.44 ± 0.42	5.80 ± 1.98	5/5	0.824 ± 0.481
DNA vaccine trial #2				
BCG	0.00 ± 0.00*	0.50 ± 0.28**	2/4	0.009 ± 0.007***
ESAT/CD80/86	1.10 ± 0.19	10.70 ± 2.32	4/4	0.220 ± 0.046
ESAT/CD154	1.85 ± 0.73	15.70 ± 5.51	4/4	1.755 ± 1.650
ESAT	1.55 ± 0.61	22.50 ± 7.80	3/4	0.838 ± 0.380

Data are presented as group mean (±S.E.M.).

[a] Lungs were subjected to a semi-quantitative pathology scoring system described previously [39]. Mean disease scores were determined by scoring each lung lobe then averaging the values to represent the entire organ for individuals within each group.

* Single asterisk indicates a significant difference ($P < 0.05$) from BCG vaccinates compared to animals receiving ESAT-6 DNA alone using Student's *t*-test.

** Double asterisk indicates a significant difference ($P < 0.05$) from BCG vaccinates compared to ESAT-6 + CD80/CD86 and ESAT-6 + CD154 DNA vaccinates using a Mann–Whitney rank sum test and indicates a significant difference ($P < 0.05$) from BCG vaccinates compared to animals receiving ESAT-6 DNA alone using Kruskal–Wallis one-way ANOVA.

*** The triple asterisk indicates a significant difference ($P < 0.05$) from BCG vaccinates compared with either ESAT-6 + CD80/CD86 or ESAT-6 + CD154 DNA vaccinates using Student's *t*-test or a Mann–Whitney rank sum test, respectively.

Table 2
Distribution of radiographic lesions per lung lobe

Vaccination group	Left cranial	Right cranial	Left caudal	Right caudal and middle	Accessory
DNA vaccine trial #1					
BCG	0.000 ± 0.000	0.000 ± 0.000	0.154 ± 0.154	0.377 ± 0.377	0.000 ± 0.000
ESAT/CD80/86	0.121 ± 0.121	0.305 ± 0.189	0.000 ± 0.000	0.143 ± 0.143	0.000 ± 0.000
ESAT	1.778 ± 1.677	1.078 ± 0.983	0.652 ± 0.404	1.042 ± 0.643	1.822 ± 1.822
Vector	1.495 ± 1.248	1.244 ± 0.909	1.165 ± 0.930	1.007 ± 0.470	0.085 ± 0.085
DNA vaccine trial #2					
BCG	0.000 ± 0.000	0.000 ± 0.000	0.009 ± 0.009	0.038 ± 0.038	0.000 ± 0.000
ESAT/CD80/86	0.086 ± 0.041	0.355 ± 0.216	0.222 ± 0.099	0.439 ± 0.162	0.000 ± 0.000
ESAT/CD154	0.937 ± 0.910	3.529 ± 3.337	2.387 ± 2.188	1.308 ± 1.248	0.616 ± 0.573
ESAT	0.232 ± 0.078	1.643 ± 1.074	1.173 ± 0.479	0.731 ± 0.429	0.409 ± 0.367

Data are presented as group mean (±S.E.M.).

Table 3
Summary of pathology in the mediastinal lymph node

Vaccination group	Mean disease score[a]	Lymph node weight (g)	CFU (ml)
DNA vaccine trial #1			
BCG	0.75 ± 0.75*	11.22 ± 3.32	186.3 ± 156.2
ESAT/CD80/86	1.00 ± 0.52*	6.65 ± 1.37***	1150.0 ± 386.2
ESAT	1.00 ± 0.58*	43.94 ± 21.66	2044.0 ± 1279.6
Vector	2.60 ± 0.20	26.30 ± 10.10	2832.0 ± 2293.6
DNA vaccine trial #2			
BCG	0.25 ± 0.25**	10.00 ± 1.34[b]	1362.5 ± 1215.7
ESAT/CD80/86	2.00 ± 0.00	14.62 ± 3.05	1395.0 ± 612.0
ESAT/CD154	2.25 ± 0.25	33.12 ± 12.65	2592.5 ± 1024.3
ESAT	2.25 ± 0.25	58.17 ± 31.38	3252.5 ± 1804.9

Data are presented as group mean (±S.E.M.).

[a] Mediastinal lymph nodes were subjected to a semi-quantitative pathology scoring system described previously [39].

[b] Indicates a significant difference ($P < 0.05$) from BCG vaccinates compared to ESAT-6 + CD154 DNA vaccinates using a pairwise comparison with a Mann–Whitney rank sum test.

* A significant difference ($P < 0.05$) from vaccinates compared to animals receiving control DNA using Student's *t*-test.

** A significant difference ($P < 0.05$) from BCG vaccinates compared to animals receiving ESAT-6 DNA alone using Student's *t*-test.

*** A significant difference ($P < 0.05$) from ESAT-6 + CD80/CD86 DNA vaccinates compared to animals receiving ESAT-6 DNA alone or vector DNA using pairwise comparisons with a Mann–Whitney rank sum test.

were homogenized for the recovery of *M. bovis* at approximately 95 days post-infection. Logarithmic dilutions of homogenates were plated onto Middlebrook 7H11 plates and incubated for 4 weeks at 37 °C, after which CFU were counted. Although not statistically significant, ESAT-6 + CD80/CD86 DNA vaccinates showed a trend towards reduced mean bacterial recovery from mediastinal lymph nodes as compared to other DNA vaccinates in trial one (Table 3). Similar results were obtained with the same cultures at 8 week (data not shown).

3.5. Cell-mediated responses to ESAT-6 DNA vaccination are enhanced with co-administration of either CD80/86 or CD154

After assessing the potential of CD80 and CD86 to augment the bovine immune response to DNA vaccination, we directly compared the adjuvant activities of CD80/CD86 to plasmid-encoded CD154 in our second vaccine trial. To determine differences in priming cell-mediated immunity, we examined the ability of bovine PBMC from different experimental treatment groups to produce IFN-γ in response to antigenic stimulation before and after aerosol challenge with *M. bovis*. Modest increases in ESAT-6-specific IFN-γ production were evident in animals receiving ESAT-6 DNA vaccines 2 weeks following boost (Fig. 2a). Animals receiving DNA vaccine co-administered with either CD80/CD86 or CD154 produced greater mean amounts of IFN-γ compared to animals receiving no costimulatory molecules. IFN-γ production was reduced 3 weeks post-boost, but was enhanced in vaccinates receiving either CD80/CD86 or CD154 following aerosol challenge with *M. bovis*. ESAT-6 + CD80/CD86 and ESAT-6 + CD154 DNA vaccinates produced approximately five- and eight-fold greater mean amounts of IFN-γ compared to animals receiving ESAT-6 DNA alone, respectively. DNA vaccination with CD154 also increased the frequency of IFN-γ producing PBMC as revealed by ESAT-6-specific ELISPOT (Fig. 2b). At 3 weeks post-boost, animals receiving CD154 DNA possessed greater mean numbers of IFN-γ spot-forming cells (SFC). Differences between and within groups were not statistically significant due to low group numbers and variation in individual responses. However, these data

suggest that DNA-encoded CD80/CD86 and CD154 may serve as adjuvants to augment IFN-γ responses to DNA vaccination.

3.6. Co-administration of CD154 does not reduce levels of pathology or bacterial titers in the lung following aerosol challenge with M. bovis

To assess disease severity, lungs were subjected to the aforementioned semi-quantitative pathology scoring system. Similar to results seen in DNA vaccine trial 1, ESAT-6 + CD80/CD86 DNA vaccinates possessed the lowest mean disease score of the lung compared with animals receiving ESAT-6 + CD154 DNA or ESAT-6 DNA alone (Table 1). The validity of these results was reinforced by enumeration of radiographic lesions from infected lungs. Radiographic analysis revealed that ESAT-6 + CD80/CD86 DNA vaccination resulted in an approximate two-fold reduction in detectable lung lesions, as compared to immunization with ESAT-6 DNA alone (Table 1). Radiographic analysis also revealed that ESAT-6 + CD80/CD86 DNA vaccinates possessed the lowest mean total percent affected lung among DNA vaccinates (Table 1). This trend was also observed on an individual lung lobe basis, with ESAT-6 + CD80/CD86 DNA vaccinates having the lowest percent affected lung on all of the lobes analyzed (Table 2). The only statistically significant differences in the lung were observed in *M. bovis* BCG vaccinates. *M. bovis* BCG vaccinates possessed significantly lower levels of percent affected lung ($P < 0.05$) compared to DNA vaccinates. Similarly, animals vaccinated with *M. bovis* BCG possessed statistically significant reductions in mean number of radiographic lesions ($P < 0.05$) compared to all DNA vaccinates, suggesting that *M. bovis* BCG vaccination is protective against *M. bovis* aerosol challenge in cattle.

Modest reductions in bacterial recovery were detected from the lungs (Fig. 3) of infected animals co-administered costimulatory molecules with ESAT-6 DNA, as compared to animals immunized with ESAT-6 DNA alone. Although not statistically significant, ESAT-6 + CD80/CD86 DNA vaccinates possessed approximately 10- and 40-fold fewer mean CFU/ml in right caudal and middle lung lobes, as compared to ESAT-6 + CD154 DNA vaccinates and animals administered ESAT-6 DNA alone, respectively. Collectively, these results suggest that although CD154 is capable of enhancing immune responses to ESAT-6, CD80/CD86 is superior in generating protective immune responses in the lung following *M. bovis* challenge.

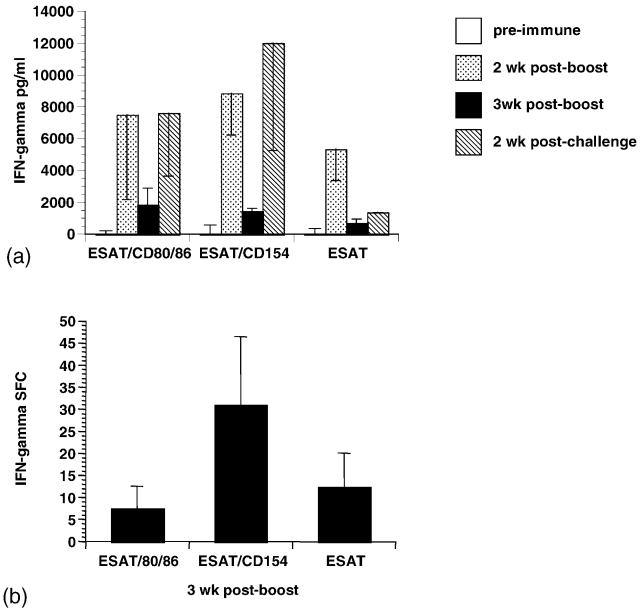

Fig. 2. ESAT-6 DNA vaccination with plasmid-encoded costimulatory molecules enhances IFN-γ responses after antigenic stimulation relative to animals immunized with ESAT-6 DNA alone. Whole blood derived from ESAT-6 + CD80/CD86 DNA vaccinates ($n = 4$), ESAT-6 + CD154 DNA vaccinates ($n = 4$) or from animals vaccinated with ESAT-6 DNA alone ($n = 4$) was stimulated for 24 h in vitro with rESAT-6 (4 μg/ml) at day 0, 2 weeks post-boost, 3 weeks post-boost and 2 weeks post-challenge. Supernatants were assayed for IFN-γ production using the BoviGam ELISA kit (a). Data represent the mean (±S.E.M.) of duplicate wells. ESAT-6 + CD154 DNA vaccinates ($n = 4$) display an increase in IFN-γ spot-forming cells (SFC) as compared to other DNA vaccinates (b). PBMC from ESAT-6 + CD80/CD86 DNA vaccinates ($n = 4$), ESAT-6 + CD154 DNA vaccinates ($n = 4$) or from animals vaccinated with ESAT-6 DNA alone ($n = 4$) was stimulated for 36 h in vitro with rESAT-6 (4 μg/ml) at 3 weeks post-boost. Data represent the mean (±S.E.M.) of triplicate cultures.

3.7. DNA vaccination with CD80/CD86 results in reduced pathology and bacterial recovery from the mediastinal lymph nodes

Following challenge, mediastinal lymph nodes were subjected to pathology scoring. ESAT-6 + CD80/CD86 DNA vaccinates possessed the lowest level of pathology amongst DNA vaccinates (Table 3). In contrast to trial one, the only

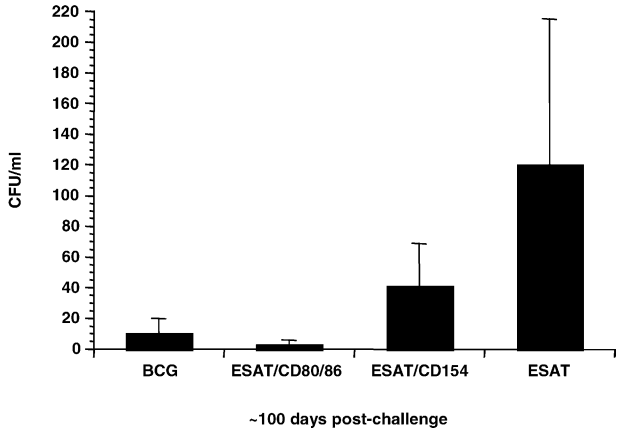

Fig. 3. ESAT-6 + CD80/CD86 DNA vaccinates ($n = 4$) display reduced bacterial titers in the lung compared to ESAT-6 + CD154 DNA vaccinates ($n = 4$). Approximately, 100 days post-challenge, right caudal and middle lung lobes were homogenized and logarithmic dilutions were plated onto Middlebrook 7H11 agar plates for 4 weeks, after which titers were determined. Data represent the mean (±S.E.M.) from animals in each treatment group.

significant difference in lymph node weights was in comparison between *M. bovis* BCG vaccinates and animals vaccinated with ESAT-6 + CD154 DNA (Table 3). To compare the protective efficacy of ESAT-6 + CD80/CD86 and ESAT-6 + CD154 DNA vaccines, mediastinal lymph nodes of infected cattle were homogenized for the recovery of *M. bovis* at approximately 100 days post-challenge. Logarithmic dilutions of homogenates were plated onto Middlebrook 7H11 plates and incubated for 4 weeks at 37 °C, after which CFU were counted. Modest, not statistically significant, reductions in bacterial recovery were detected from the mediastinal lymph nodes (Table 3) of infected animals co-administered CD80/CD86 with ESAT-6 DNA, as compared to animals immunized with ESAT-6 DNA alone. Bacterial recovery was reduced in mediastinal lymph nodes with ESAT-6 + CD80/CD86 DNA vaccinates possessing an approximate two-fold reduction in mean bacterial titers compared to other DNA vaccinates. Similar results were obtained with 8 week cultures (data not shown).

4. Discussion

The activation of naïve T lymphocytes is known to require at least two signaling events to occur. The first signal involves TCR recognition of a foreign antigen presented by a MHC molecule. The second signal is mediated through a costimulatory receptor. In naïve T cells, this signal is primarily received through CD28-CD80/CD86 interactions [41–43]. The combination of these signals drives T cells into the cell cycle, induces differentiation and augments cytokine production, leading to an effective immune response [41–43]. Previous studies conducted in mice have shown that immune responses to DNA vaccination can be improved with the co-administration of costimulatory molecules [16–20,25,26]. Additionally, CpG ODN have been shown to possess intrinsic adjuvant abilities that induce immune responses in humans, cattle and rodents [13]. Our study explores for the first time, the adjuvant effects of plasmid-encoded CD80 and CD86 versus CD154 on DNA vaccine-induced immunity in cattle.

Our first DNA vaccine trial with CpG ODN, ESAT-6 and CD80/CD86 resulted in a marked enhancement of immune responses, assessed by IFN-γ production, lymphocyte proliferation and reduced pathology following aerosol challenge with virulent *M. bovis*. Notably, the results from this first trial suggest that immunity can be generated in large animals by a single prime/boost vaccination regimen. ESAT-6 DNA delivered in the absence of CD80 and CD86 generated similar immune responses; however, major differences were seen upon post-mortem examination with ESAT-6 DNA vaccinates possessing more severe pathology and greater relative lung lesion area. ESAT-6 + CD80/CD86 DNA vaccination also resulted in reduced bacterial recovery from mediastinal lymph nodes, as compared to animals vaccinated with ESAT-6 DNA alone or control cattle. Bacterial recovery from the lung was not significant at the dose and timepoint used in trial one.

After evaluating the use of CD80/86 as molecular adjuvants, we next conducted a similar DNA vaccine trial to compare adjuvant abilities of CD80/CD86 with those of CD154. Previous studies have determined that DNA vaccination with CD154 can enhance immune responses in rodents and large animals [12,25,26]. CD154 interactions with its receptor, CD40, on APC have been shown to result in the upregulation of costimulatory molecules, namely B7 molecules, as well as the production of pro-inflammatory cytokines, such as IL-12 [23,24]. Vaccination with plasmid-encoded CD154 generated enhanced IFN-γ responses to ESAT-6 following boost and challenge timepoints. Surprisingly, following challenge, ESAT-6 + CD154 DNA vaccinates possessed greater levels of pathology in the lung and higher bacterial titers in the lung and mediastinal lymph node when compared to animals receiving ESAT-6 + CD80/CD86 DNA. Comparisons between control immunized animals were excluded in vaccine trial two as the infection was uncharacteristic of non-vaccinated cattle. In both trials, in addition to statistically significant differences, modest differences were observed in the several measured parameters that resulted in a clear trend towards protection. Larger differences may become evident with greater numbers of animals within groups, but small group numbers are an inevitable limitation of large animal experiments that require high level bio-containment. Regardless, these differences suggest that, in cattle, CD80/86 is superior to CD154 in augmenting DNA vaccine-induced protection despite the induction of enhanced IFN-γ responses to recall stimulation with rESAT-6.

Presumably, the combined action of our ESAT-6 + CD80/CD86 DNA vaccine is to enhance the activation of naïve T lymphocytes, resulting in an effector pool of T cells capable of controlling infection with virulent *M. bovis*. In DNA vaccination, plasmid DNA is internalized and expressed by two main cells types: dendritic cells and muscle cells. It has been shown previously that muscle cells expressing costimulatory molecules cannot prime T cell responses [44], suggesting that DC are the key mediators of DNA vaccine-induced immune responses. However, muscle cells may present antigen to activated T cells or produce exogenous antigen for DC to internalize and cross-present [45,46]. Previous work conducted in our laboratory has shown that immature bovine DC do not constitutively express relatively high levels of CD80, but do express relatively high levels of CD86, suggesting that plasmid-encoded costimulatory molecules may represent a means to activate T cells more efficiently [34]. We observed an enhancement of immune responses to ESAT-6 by DNA vaccinates immunized with either CD80/CD86 or CD154; however, CD154 immune responses did not correlate with enhanced protection against tuberculosis. A recent report revealed that $CD4^+$ and $CD8^+$ T cell responses against *M. tuberculosis* occur normally in the absence of CD154, suggesting alternative ligands for CD40 [47]. Other infections,

with microbes such as *Listeria monocytogenes* [48] and lymphocytic choriomeningitis virus [49], have shown that $CD8^+$ T cell priming occurs independently of CD40/CD154 interactions. Demangel et al. [50] found that mice immunized with BCG-infected CD40-stimulated DC had enhanced immune responses to *M. tuberculosis* infection; however, this enhancement did not correlate with increased protection following infectious challenge, suggesting that additional strategies were needed. These studies also demonstrated that stimulation via CD40 also resulted in increased expression of IL-10 mRNA in vitro [50] and that autocrine expression of IL-10 in vivo impaired DC responses to mycobacteria [51]. The basis for the effects of CD154-enhanced IFN-γ responses, but reduced elimination of bacteria and increased pathology observed in our vaccination trial will require further study.

MHC class I-restricted $CD8^+$ CTL have been shown to have a role in protection against tuberculosis in animals and man [27]. DNA vaccination is an ideal strategy to target these T cells, as endogenous antigen, produced by cells that internalize plasmid DNA, is presented via MHC class I molecules [52]. One model regarding the generation of immunologic memory hypothesizes that only a brief encounter with antigen is necessary for the formation of $CD8^+$ effector/memory T lymphocytes and that a programmed division occurs independently of further antigenic stimulation, resulting in a stable pool of memory cells [53,54]. Therefore, a sufficient priming event may be capable of generating an effector pool of activated T cells able to control or eradicate infectious agents. CTL generated in response to tuberculosis may directly kill infected cells through the secretion of the anti-microbial protein, granulysin [55]. Granulysin has been shown to possess this activity against a broad spectrum of bacteria in vitro by inducing membrane instability [55]. This protein has been identified in humans and recent work in our laboratory has identified a homologue for this gene in cattle [56].

In conclusion, our data suggest that the co-administration of CD80 and CD86, but not CD154, enhances DNA vaccine-induced protection to experimental bovine tuberculosis, particularly at the level of lung and lymph node pathology. Furthermore, we have established that the addition of these costimulatory molecules enables immunity to be generated in cattle following a single prime/boost regimen. Additionally, we have shown for the first time that *M. bovis* BCG vaccination is protective against experimental aerosol challenge with virulent *M. bovis*. To our knowledge, this study represents the first large animal experiments to examine the effects of plasmid-encoded CD80/CD86 on DNA vaccination, as well as a direct comparison with CD154. Currently, we believe that the co-administration of CpG ODN with plasmid-encoded CD80 and CD86 may represent a practical vaccine platform for use in large animals, capable of generating robust, long-lived immune responses, which may also be applicable to the prophylaxis of other bacterial, viral and parasitic diseases.

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